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Purification of Extracellular Vesicles From Mesenchymal Stromal Cells Grown in 3D Culture

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Abstract

Extracellular vesicles (EVs) are biological carriers that play a crucial role in intercellular communication, making them promising candidates for delivering therapeutic material to cells. Robust, scalable, and well-characterized production methods are essential to realize their full clinical potential.

In this application note, we present an end-to-end process for the production and purification of EVs from human bone marrow-derived mesenchymal stromal cells. The established method addresses challenges related to scalability and reliable analytics, providing a robust foundation for the clinical-scale production of EVs. This comprehensive approach ensures high-quality EV production, paving the way for their application in therapeutic and diagnostic fields.

Introduction

Extracellular vesicles (EVs) are small, membrane-bound nanoparticles secreted by cells into the extracellular environment. They typically range from 30 to 1,000 nm and carry a diverse payload of proteins, lipids, nucleic acids, and other bioactive molecules. EVs play crucial roles in intercellular communication, shuttling biological information between cells and modulating various physiological and pathophysiological processes. They engage in immunomodulation, tissue repair, and disease progression, including cancer metastasis. Their diverse cargo and ability to traverse biological barriers make EVs promising candidates for diagnostic and therapeutic applications.[1] Despite this growing interest, there are numerous challenges in generating high-quality EVs, including implementing scalable solutions for upstream production and downstream purification.[2] In addition, robust analytical characterization methods using multiple orthogonal techniques, including single particle and analytical chromatography technologies, are critical to providing a complete profile of extracellular particles collected in both upstream and downstream processing.

In this application note, we describe the development of a scalable end-to-end process to produce and characterize high-quality EVs from human bone marrow-derived mesenchymal stromal cells (hBM-MSCs) grown in 3D culture.

Materials and Methods

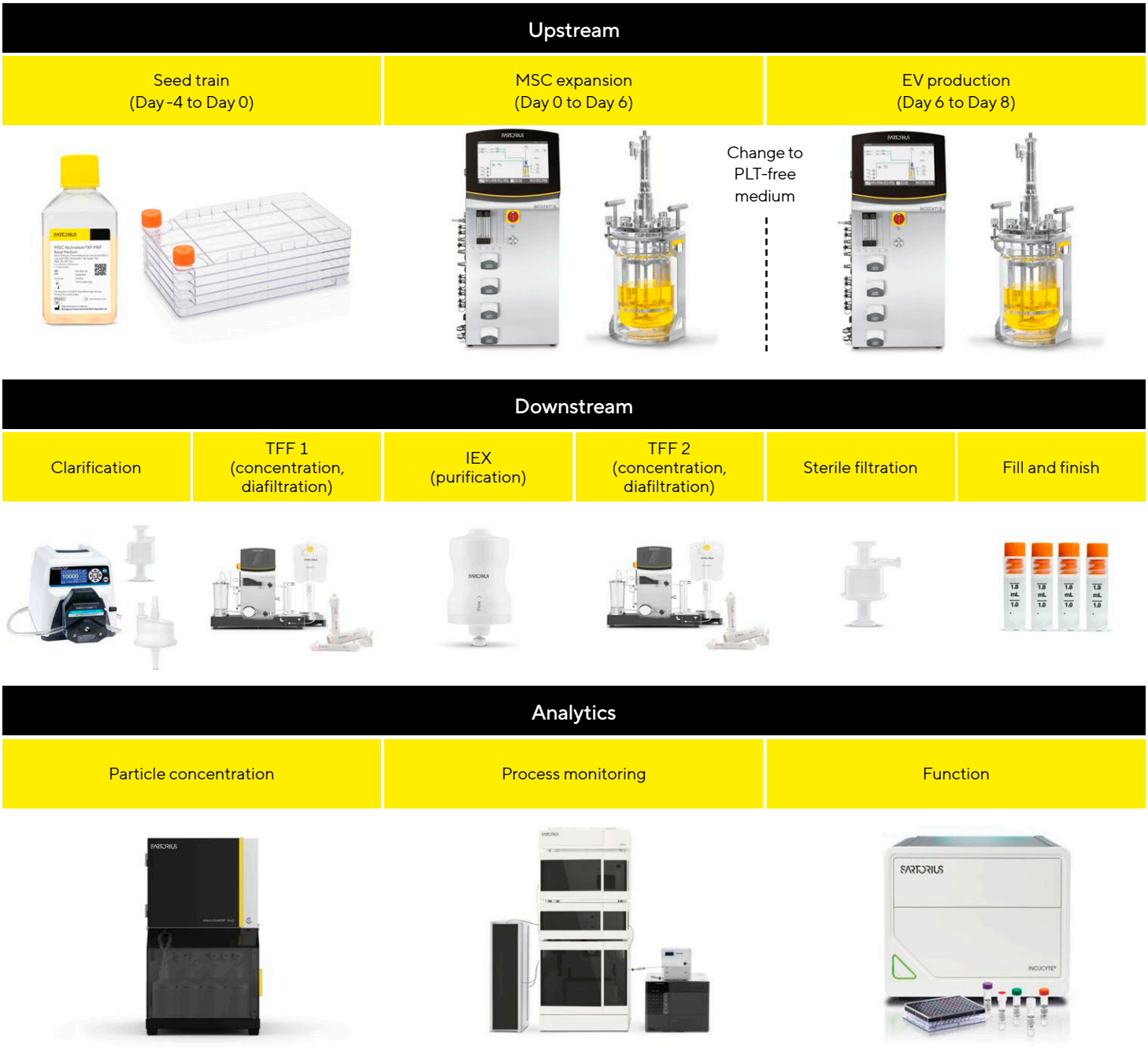
Process overview

We have developed a scalable process using a diverse range of Sartorius equipment and consumables to produce high-quality EVs from hBM-MSCs. Cells were grown in 3D culture until confluent. Human platelet lysate (PLT) was removed from the medium at the harvest stage, and the medium was conditioned. The conditioned medium containing EVs was clarified and then processed using tangential flow filtration (TFF) and chromatography. The process is outlined in Figure 1. After the EVs were purified, they were characterized using multiple analytical techniques.

Cell culture

hBM-MSCs were initially cultured on a 10-stack tissue culture dish (Thermo Fisher Scientific) at 37 °C and 5% CO_2. The cells were plated at 3,000 cells/cm² in MSC NutriStem® XF basal medium (Sartorius) supplemented with MSC NutriStem® XF supplement (Sartorius) and PLTGold® Human Platelet Lysate (clinical grade; Mill Creek Life Sciences). Once confluent, they were dissociated using TrypLE™ Express (Thermo Fisher Scientific) and added to a Univessel® Glass 2 L bioreactor with Biostat® B (Sartorius) containing 10 cm²/mL rhCol1-coated SoloHill® ACF microcarriers (10 cm² × 2,000 mL medium ÷ 360 cm²/g = 55.6 g of microcarriers; Sartorius) in complete medium at 3,000 cells/cm². The attachment of cells to microcarriers and cell growth was observed daily.

Figure 1: Schematic of the end-to-end EV purification process



Note. IEX = ion-exchange chromatography

The agitation speed was initiated at 45 rpm and increased daily as cell-microcarrier aggregation occurred. On day three, the agitation was stopped, and the microcarriers were allowed to settle to the bottom of the bioreactor, after which 50% of the medium was removed and replaced with fresh complete medium and the agitation restarted. On day six, the agitation was stopped, and the microcarriers were allowed to settle to the bottom of the bioreactor, after which 80% of the medium was removed and replaced with dPBS to wash the MSC-coated microcarriers and remove PLT. This process was repeated for a total of two washes.

The MSC-coated microcarriers were then resuspended in 1,800 mL of MSC NutriStem® XF basal medium supplemented with MSC NutriStem® XF supplement (EV collection medium). Agitation was resumed and the culture was continued for an additional two days to allow for EV production and secretion into the medium. The EV-conditioned medium was harvested and used for subsequent downstream purification. Additional culture parameters are outlined in Table 1.

Table 1: Culture parameters

Parameter	Settings
Cell type	hBM-MSC
Seeding density	3,000 cells/cm²
Base medium	MSC NutriStem® XF
Microcarrier type	SoloHill® rhCol1-coated ACF (prototype)
Microcarrier density	10 cm²/mL
Medium maintenance	Batch (50% medium exchange)
Growth duration	6 days
EV conditioning	2 days
Temperature	37 °C
Agitation speed	45 – 130 rpm (ramp)
pH	7.35
Dissolved O_2	50%
Gas delivery	Overlay

Cell count, viability, and attachment

Daily sampling to measure cell count and viability on microcarriers was performed by taking a 10 mL sample from the bioreactor each day. The sample was spun at 300 × g for 5 min, and the medium was removed. The pelleted material was washed with dPBS and spun at 300 × g for 5 min. The dPBS was removed and the pelleted material was dissociated using 2 mL of TrypLE™ Express. After 5 min, the reaction was quenched with 4 mL of dPBS and passed through a 70 µm cell strainer (Corning) to remove the microcarriers. Concentration and viability were determined using a NucleoCounter® NC-200™ cell counter (ChemoMetec). Cells on microcarriers were imaged on a Nikon Eclipse Ti microscope.

Clarification

A two-step clarification strategy was used to remove residual microcarriers and cell debris. Conditioned medium containing EVs was pumped via a peristaltic pump (Masterflex) through a 5 µm Sartopure® PP3 filter (capsule size 5, 0.036 m^2; Sartorius) at a rate of 100 mL/min (167 LMH). The 5 µm filtered medium was then pumped through a 0.65 µm Sartopure® PP3 filter (size 5, 0.026 m^2) at a rate of 100 mL/min (231 LMH). Both filters were conditioned with dPBS prior to processing conditioned medium. The clarified medium was stored at 4 °C.

TFF 1

Clarified medium containing EVs was processed using the Sartoflow® Smart TFF system (Sartorius) with a 12-inch, 0.5 mm lumen and 750 kDa WaterSep® Explorer (Sartorius) hollow fiber filter. A shear rate of approximately 8,000 s^{-1} was chosen to achieve optimal results.

The feed pressure was set at 1 bar, while the permeate pressure was set between 0.05 and 0.1 bar. Once the conditions were met, the medium was concentrated fivefold at room temperature. After concentration, the system was stopped, and Benzonase® (Merck Millipore) was added at a concentration of 50 U/mL for 1 hour to remove any contaminating DNA. After the Benzonase® treatment was completed, the medium was diafiltered into chilled dPBS for 3 diafiltration volumes (DVs). The volume of the retentate was held constant by an auxiliary pump controlled by the machine that pumped the buffer into the system using the same conditions as described above.

Ion-exchange chromatography

Prior to ion-exchange (IEX) chromatography, 250 mM NaCl was added to the concentrated EV solution to prevent unwanted protein interactions. EVs were purified from this solution using a 4 mL CIMmultus® QA monolith (Sartorius) connected to an ÄKTA avant™ 150 chromatography system (Cytiva) at a rate of 5 mL/min. The mobile phase buffer was 50 mM Tris pH 7.5, and the gradient buffer was 50 mM Tris pH 7.5, 2,000 mM NaCl. After an initial 10-column volume (CV) equilibration with 5% gradient buffer (50 mM Tris pH 7.5, 100 mM NaCl), the EV medium was added, and the flowthrough was collected. The column was washed with 18 CVs of 5% gradient buffer, and 50 mL fractions were collected. The elution was performed using a linear gradient from 5 to 100% with gradient buffer (50 mM Tris pH 7.5, 100 mM NaCl to 50 mM Tris pH 7.5, 2,000 mM NaCl), and four 24 mL fractions were collected. All fractions were stored at 4 °C until analysis. A clean-in-place (CIP) step using 1 M NaOH, 2 M NaCl was used to strip the column, which was then re-equilibrated with a 5% gradient buffer. EV fractions corresponding to the OD280 peak with conductivity between 45 – 125 mS/cm (500 – 1,500 mM NaCl) were pooled for TFF.

TFF 2

The pooled fractions from IEX were added to the Sartoflow® Smart system, which contained the same setup and parameters as the first TFF concentration step. The pooled fractions were diafiltered into chilled dPBS for 3 DVs and then concentrated approximately threefold for a final volume of 45 mL.

Final sample filtration

The concentrated preparation of purified EVs was syringe-filtered through a 0.8 | 0.2 µm Sartopore®2 XLG 25 mm filter (Sartorius). The EVs were then aliquoted into 1 mL aliquots and stored at -80 °C.

Bradford protein assay

Samples and bovine serum albumin (BSA) standards (31.25 – 2,000 µg/mL) were diluted as needed. Samples (10 µL) were added to a clear 96-well plate, and 300 µL of Bradford reagent (Thermo Fisher Scientific) was added. The plate was incubated at room temperature for 5 min and then read at an optical density of 595 nm using a SpectraMax® M2e plate reader (Molecular Devices). The sample ODs were interpolated off the standard curve using 4-PL curve fitting to obtain the protein concentration.

PicoGreen™ DNA assay

Residual DNA was quantified using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, DNA standards (0.5 ng/mL – 1,000 ng/mL) were prepared and 100 µL of each sample and standard were added to a black 96-well plate, followed by the addition of 100 µL of working PicoGreen™ reagent. The plate was incubated in the dark at room temperature for 3 min and then read at an excitation of 480 nm and emission of 520 nm using a SpectraMax® iD3 plate reader (Molecular Devices). The DNA content of the samples was interpolated from the standard curve using linear fitting.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to assess the concentration and particle size of the purified EV preparations. The NanoSight® NS300 (Malvern) was determined to have an optimal particle concentration range between 0.8 × 107 and 8 × 108 particles/mL. For these studies, a concentration corresponding to around 30 particles/frame was used to evaluate the preparations. The EV preparations were diluted in dPBS and loaded into the chamber of the NanoSight®. The filter was set to light scatter mode, and the focus was adjusted so that the particles could be clearly visualized. The camera level was set to 14, and the sample was injected using a flow rate of 30. The script was executed to capture five 30-second videos. Once the videos were captured, they were analyzed with a detection threshold set to level 5. For fluorescent NTA (FI-NTA), samples were labeled with a plasma membrane stain, CellMask™ Orange (Invitrogen), for 30 min at room temperature. Then, NTA was performed as described in fluorescent mode with a 532 nm laser module.

Flow cytometry

Virus Counter® 3100 (Sartorius) is a nanoflow cytometer that was used to orthogonally evaluate particle concentration. Samples were labeled with CellMask™ Orange, tested using FI-NTA, and then diluted 10 × and run directly on the instrument. For each test, the sample was run through the flow cell of the instrument equipped with a 532 nm laser line at 150 mW laser power. Three independent replicates of each sample were tested for 60 seconds following the manufacturer's software manual. A custom MATLAB® script was used to quantify voltage peaks and particle concentration. For lysing, purified EV samples were treated with 2% sodium dodecyl sulfate for 30 min, diluted, and measured on the Virus Counter®.

Analytical size-exclusion chromatography

Analytical size-exclusion chromatography (SEC) was performed using the PATfix® analytical platform (Sartorius) with a Bio SEC-3, 300 Å, 7.8 × 300 mm analytical column with a resolution from 1.25 – 5 kDa (Agilent). An AdvanceBio® SEC size standard (Agilent) was used to determine the retention time of particles as they pass through the column. Samples were diluted in PBS as needed, placed in vials, and 50 µL was injected into the machine at a flow rate of 0.5 mL/min for 35 min. Larger particles were eluted first, followed by increasingly smaller particles. UV absorbance at 260 nm and 280 nm, fluorescence at 280 nm excitation and 350 nm emission (intrinsic fluorescence), and multi-angle light scatter (MALS) at 60° and 90° detectors were used to assess each sample's characteristics.

Cryo-electron microscopy

For cryo-electron microscopy (Cryo-EM), samples were diluted in PBS as needed and preserved in vitrified ice supported by holey carbon films on 400-mesh copper grids. Each sample was prepared by applying a 3 µL drop of sample suspension to a cleaned grid, blotted away with filter paper, and immediately followed with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using a Glacios™ Cryo-Transmission Electron Microscope (Thermo Fisher Scientific) operated at 200 kV and equipped with a Falcon 4 direct electron detector. Vitreous ice grids were clipped into cartridges, transferred into a cassette, and then into the Glacios™ autoloader, all while maintaining the grids at cryogenic temperature (below –170 °C). Automated data collection was carried out using Leginon software, where high-magnification movies were acquired by selecting targets at a lower magnification. Images of each grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnifications, high-magnification images were acquired at nominal magnifications of 73,000 × (0.204 nm/pixel) and 28,000 × (0.512 nm/pixel). The images were acquired at a nominal underfocus of –7.0 µm to –4.5 µm and electron doses of ~2 – 12 e–/Å^2.

Capillary electrophoresis-based immunoassay

Immunoassays (Western blot) were performed using the Jess Simple Western™ instrument (ProteinSimple) according to the manufacturer's guidelines. Briefly, sample protein content was determined as previously described, and samples were diluted in 0.1 × sample buffer to a total protein concentration of 0.4 µg/µL. Then, four parts sample were mixed with one part 5 × fluorescent master mix containing 5 × sample buffer, fluorescent standard, and 200 mM DTT from the EZ standard pack (ProteinSimple). Proteins were denatured at 95 °C for 5 min. MSC lysates were used as positive controls. Antibodies were diluted in antibody diluent #2, and a Jess separation module with 12 – 230 kDa range was used according to the manufacturer's guidelines. Briefly, prepared samples, primary antibodies, secondary antibodies, biotinylated ladder, streptavidin-HRP, Luminol-S, and peroxide mixtures were loaded on a microplate and the chemiluminescence assay was set as follows: separation time = 30 min, separation voltage = 375 V, antibody diluent time = 30 min, primary antibody time = 30 min, secondary antibody time = 30 min. The chemiluminescent detection profile was set at High Dynamic Range 4.0 and contrast was manually adjusted for each sample.

Proteomics

Purified EV samples were prepared for proteomics by adjusting buffer composition to 8 M urea, pH 8.0, with 1 × cOmplete™ Protease Inhibitor (Roche Diagnostics). Samples were sonicated using an amplitude of 35 – 40% with 1 s pulses for 20 s. Lysates were clarified by centrifugation at 14,000 × g and supernatants transferred to a new tube. Protein quantitation was performed using Qubit™ (Thermo Fisher Scientific). 50 µg of sample was digested overnight with trypsin. Briefly, samples were reduced for 1 h at room temperature in 12 mM DTT followed by alkylation for 1 h at room temperature in 15 mM iodoacetamide. Trypsin was added at an enzyme:substrate ratio of 1:20 for 18 h. Each sample was acidified to 0.3% TFA and subjected to solid-phase extraction using Waters µHLB (Waters). Briefly, the matrix was activated with four additions of 500 µL of 70% acetonitrile. Then, it was equilibrated with four additions of 500 µL of 0.3% TFA. Sample was added to each well, and the wells were washed with three additions of 500 µL of 0.3% TFA. Samples were eluted in 200 µL of 60% acetonitrile in 0.3% TFA before eluting in an additional 400 µL of 60% acetonitrile in 0.3% TFA. The samples were frozen at –80 °C and lyophilized overnight.

The peptides were reconstituted in 0.1% TFA. For mass spectrometry, 500 ng per sample was analyzed by liquid chromatography-mass spectrometry (LC-MS) with a Vanquish™ Neo UPLC system interfaced to an Orbitrap™ Astral™ (Thermo Fisher Scientific). Peptides were loaded on a trapping column and eluted over a 75 µm analytical column at 350 nL/min; the column was heated to 55 °C. A 30 min gradient was employed. The mass spectrometer was operated in data-independent mode. Sequentially, full-scan MS data (240,000 FWHM resolution) from m/z 380 – 980 was followed by 300 × 2 m/z precursor isolation windows; products were acquired in the Astral™ at 40,000 FWHM resolution. The maximum ion inject time (IIT) was set to 3.5 ms for data-independent acquisition (DIA); the normalized collision energy was set to 25. DIA data were analyzed using DIA-NN (v1.9.1) to convert RAW files to QUANT, align based on retention times, search data using an in silico spectral library created from the FASTA file and iteratively from the spectral library created from RAW data, filter the database search results at 1% peptide and protein false discovery rate (FDR), calculate the peak areas for detected peptides, and normalize the data. Data were searched with the Swiss-Prot Human database, peptide FDR = 0.01, and protein FDR = 0.01. A total of 4,581 proteins were detected. The heatmap represents three technical replicates of a purified EV sample.

EV uptake

A549 cells were expanded and plated in a 96-well plate at 2.5×10^4 cells/well in F12 medium containing 10% FBS. Purified EVs were labeled using the Incucyte® Exofluor Green EV Labeling Kit (Sartorius) at 80 µM and 37 °C for 30 min. After labeling, the mixture was transferred to a Vivaspin® 2 protein concentrator column (Sartorius) and centrifuged at 2,000 × g for 5 min. The samples were washed with PBS once and collected in particle-free medium. Fluorescently labeled purified EVs or dye-only controls were added to the cells and monitored via the Incucyte® Live-Cell Analysis System and analyzed via the Incucyte® Adherent Cell-by-Cell Analysis Software Module (Sartorius).

EV wound closure

An Incucyte® Imagelock 96-well Plate (Sartorius) was coated with 50 µL per well of rat tail collagen I for 2 h at room temperature. Normal human dermal fibroblasts were expanded before being added in DMEM + 10% FBS at 15,000 cells/well and allowed to adhere overnight. An Incucyte® 96-Well WoundMaker Tool (Sartorius) was used according to a standard protocol to create uniform wounds across the collagen-coated | seeded assay plate. After wounding, medium was aspirated and cells were washed twice with 100 µL of assay medium (serum-free DMEM). After rinsing, 100 µL of fresh assay medium, with or without EVs, was added. The plate was analyzed in the Incucyte® Live-Cell Analysis System using the Incucyte® Scratch Wound Analysis Software Module (Sartorius) to quantify the EV-mediated wound closure. The data are presented as the average and standard error of six wells.

Results and Discussion

Cell growth

The growth and viability of cells on microcarriers were measured using daily sampling to obtain cell counts from a representative bioreactor sample (Figure 2). The growth of the cells was also monitored visually with brightfield and fluorescence microscopy using DAPI staining of nuclei to detect cells on the surface of the microcarriers (Figure 2). On day three, aggregation of microcarriers was observed due to cell bridging. Consequently, the agitation speed was increased to ensure the cell-laden microcarriers remained in suspension.

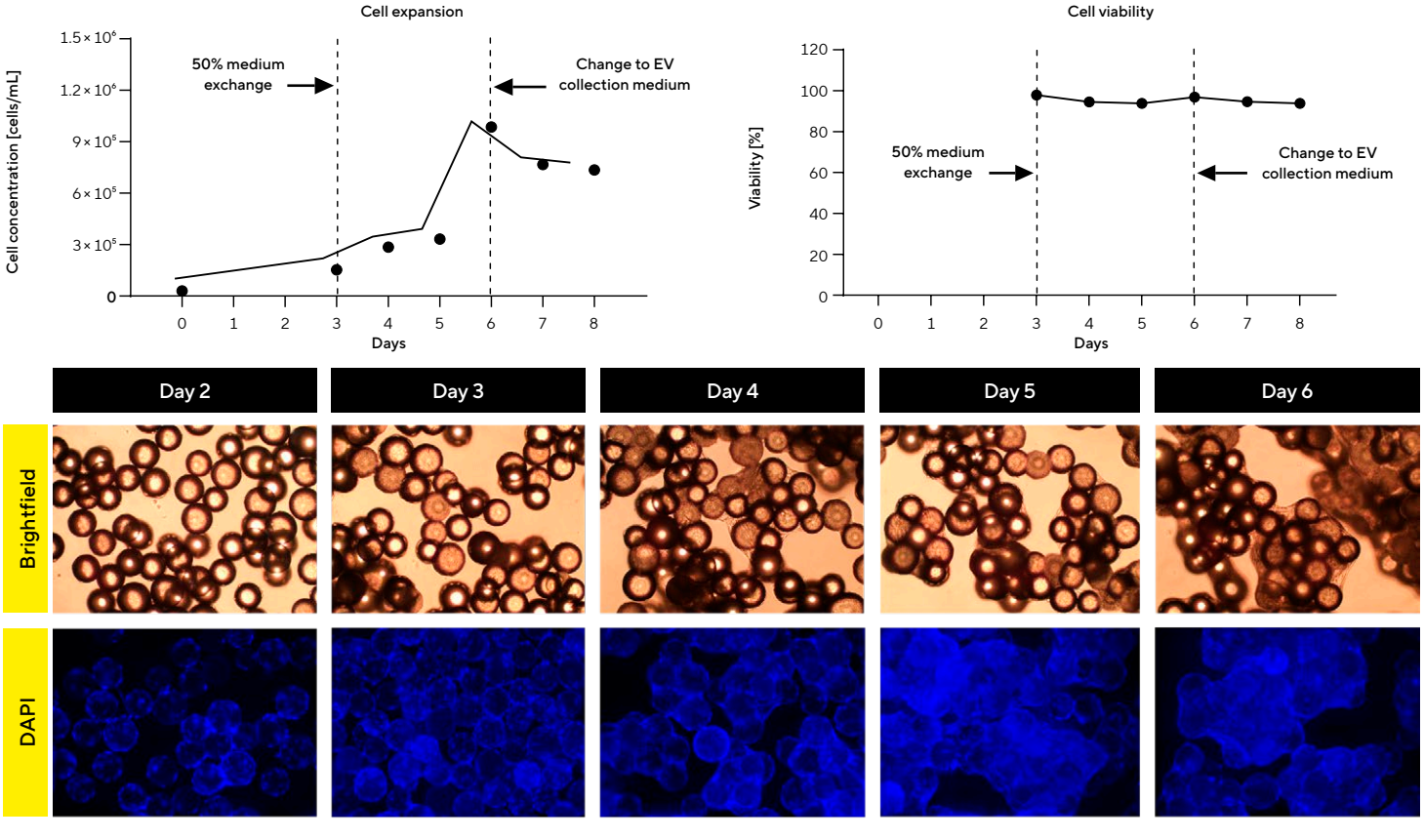
Glucose levels were also monitored (data not shown), and on day three, a 50% batch medium exchange was performed to replenish the depleted medium. After the addition of the fresh complete medium, the cell numbers increased substantially and reached a concentration of approximately 0.75×10^6 cells/mL (Figure 2). On day six, the medium was exchanged for fresh medium without PLT (EV collection medium) to ensure that the EVs collected in the medium were produced by the MSCs. The medium was conditioned for two days, and on day eight, the medium was harvested. Across the entire process, cell viability remained above 90% (Figure 2).

Downstream process analysis

The strategy for processing the cell-conditioned medium was to maximize EV retention while reducing contaminants, including large cell debris, proteins, and nucleic acids. The removal of protein contaminants across each unit operation of the downstream process was monitored by the Bradford assay, and the removal of DNA contaminants was monitored by the PicoGreen™ assay (Figure 3). The conditioned medium was collected from the bioreactor and clarified using a two-step process to remove cell debris. An initial 5 µm filtration step removed large debris, after which the medium was filtered through a 0.65 µm filter to remove smaller debris. After clarification, the medium was visually clear.

TFF was then performed to concentrate and diafilter the medium. The medium was treated with 50 U/mL Benzonase® between TFF concentration and diafiltration to remove DNA. After diafiltration, 51% of the total protein and 80% of the DNA was removed. Before chromatography, 250 mM NaCl was spiked into the sample to help eliminate lower affinity contaminating proteins from binding to the IEX monolith. Albumin contained within the medium has been shown to have low affinity above a conductivity of around 20 – 30 mS/cm (200 – 300 mM NaCl).

Figure 2: MSC expansion in the bioreactor (top) and brightfield and DAPI images showing cell bridging over time (bottom)



After NaCl addition, the sample was further purified using a CIMmultus® QA column. EVs were eluted using a linear NaCl gradient, and fractions containing EVs were collected. A second TFF step was used to diafilter the pooled fractions into dPBS and ultimately concentrate the purified EVs. Finally, the EVs were filtered through a 0.8 | 0.2 µm syringe filter, and aliquots were frozen at –80 °C. Analysis of the final sample showed a 545-fold reduction in protein, resulting in 99.8% of the total protein being removed during purification. Similarly, 99.4% of DNA was removed (Figure 3). Furthermore, particle recovery was monitored after major unit operations using three orthogonal analytics techniques: scattering NTA (Sc-NTA), Fl-NTA, and Virus Counter®. Sc-NTA showed 27%, Fl-NTA 28%, and Virus Counter® 33% particle recovery in the final sample (Figure 3).

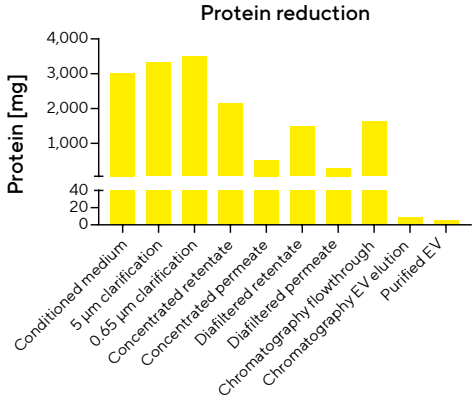
Downstream process analysis using analytical SEC

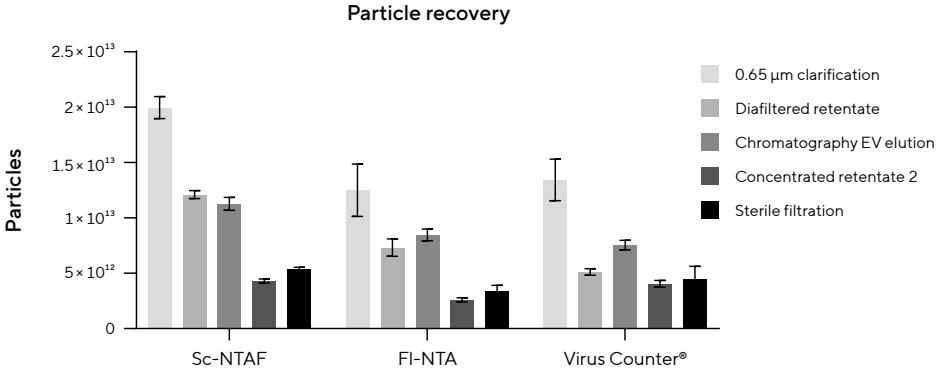
The PATfix® analytical system with in-line MALS, UV, and fluorescence detectors was used to determine EV and impurity composition after major unit operations. In general, EVs are expected to exhibit relatively high MALS and low UV and fluorescence signals while free proteins and nucleic acids exhibit relatively high UV and fluorescence and low MALS signals.[3]

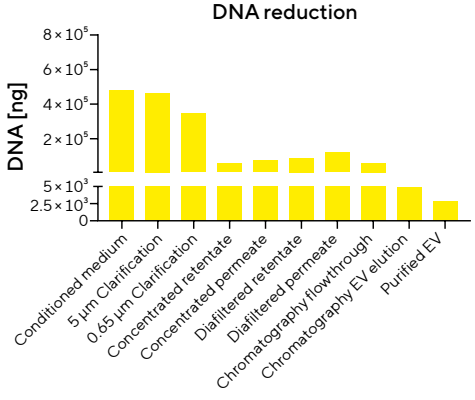
Elution windows based on EV particle size, large impurities (14–20 min), and small impurities (20–30 min) are highlighted in Figure 4.

PATfix® revealed that TFF concentration increased the signal for EV-sized particles and large impurities while greatly reducing small impurity signals. Furthermore, IEX greatly concentrates EV-sized particles, as shown by increased MALS signal, while virtually removing large and small impurities. Because TFF diafiltration removes the MALS signal in the small impurity region, this signal is likely an artifact of the IEX buffer. Although there is a small decrease in EV-sized particle MALS signal with TFF diafiltration, there is virtually no loss with sterile filtration (Figure 4). These data agree with total protein and DNA reduction in Figure 3, as well as with particle recovery data. Thus, PATfix® enables detailed compositional analysis and assessment of sample purity through size-based separation coupled with UV, fluorescence, and MALS detectors.

Figure 3: Downstream process analysis of impurity removal and particle recovery







Parameter		Conditioned medium	Purified MSC-EVs	Reduction | recovery
Protein [mg]		3018	5.45	[99.8%] reduction
DNA [ng]		478494	2844	[99.4%] reduction
Particles	Sc-NTA	2.00×10^{13}	5.39×10^{12}	[27%] recovery
	Fl-NTA	1.25×10^{13}	3.45×10^{12}	[28%] recovery
	VC	1.34×10^{13}	4.48×10^{12}	[33%] recovery

Figure 4: Downstream process analysis using analytical SEC

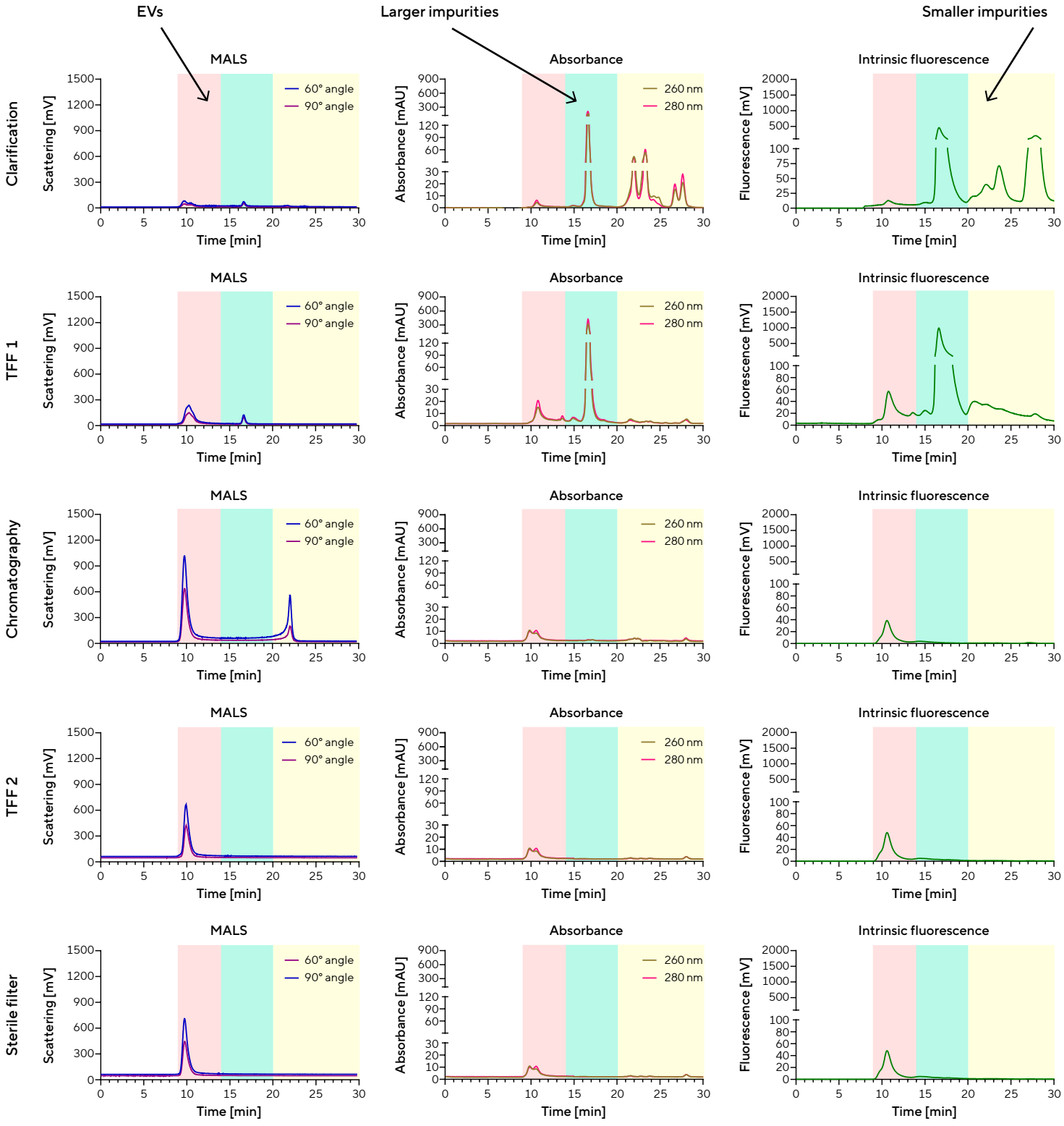
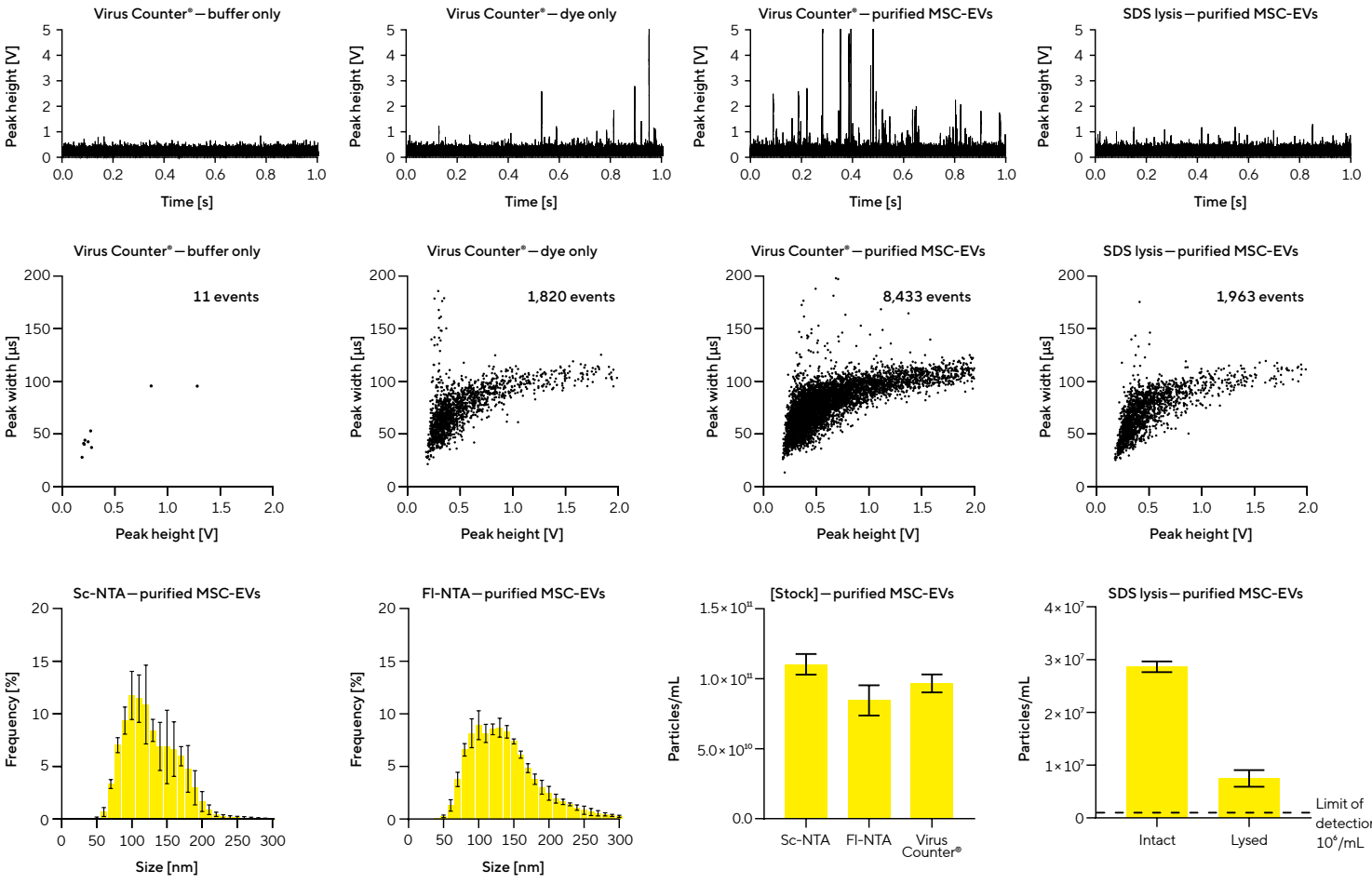


Figure 5: Final product characterization using Virus Counter® and NTA

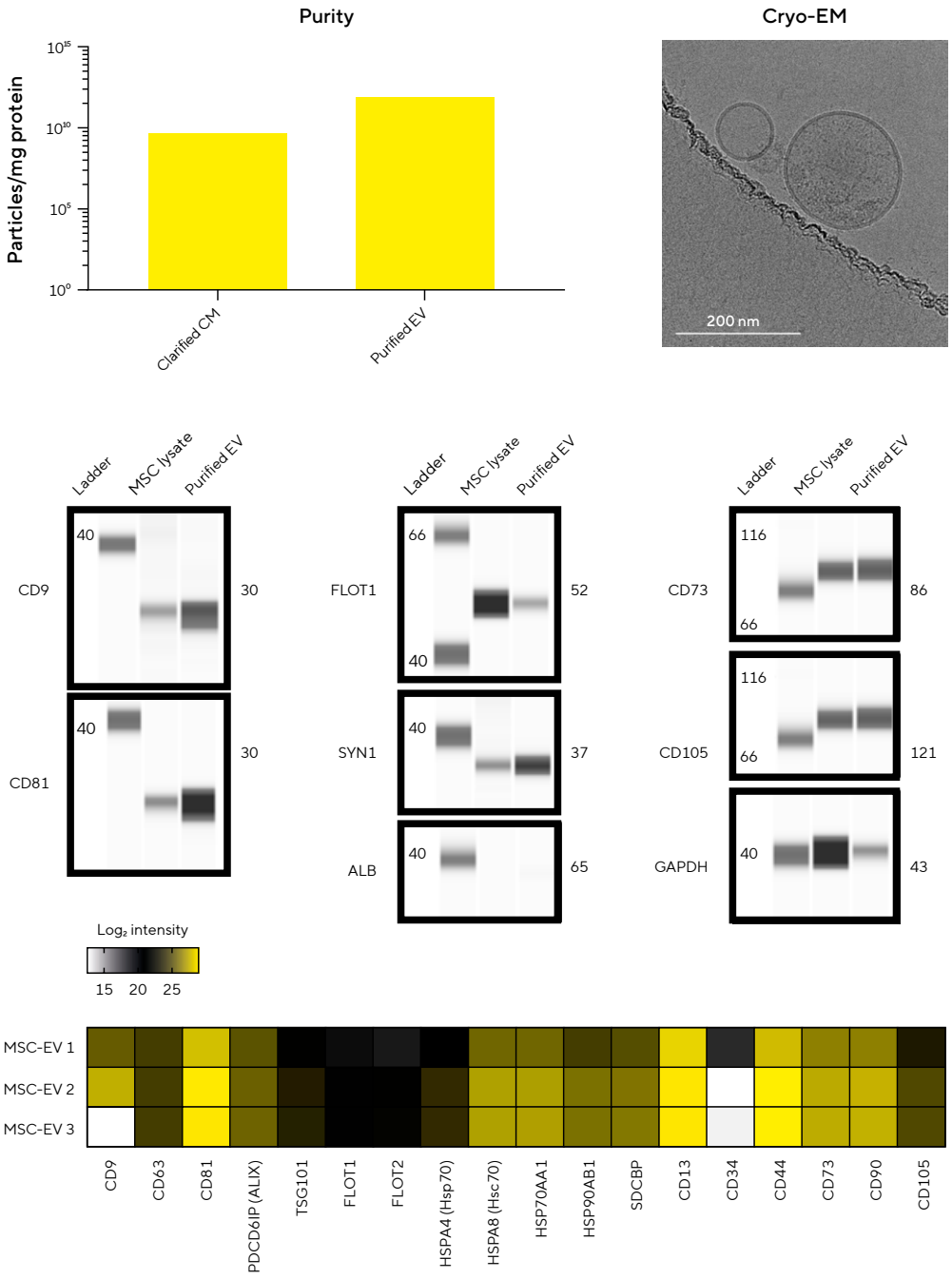


Final product characterization

First, Virus Counter® and NTA were used to determine particle concentration and size in the purified EV sample. The buffer-only sample, consisting of dPBS, showed virtually no peaks and thus no events in Virus Counter®. Furthermore, dye-only (CellMask™ Orange) samples in buffer showed 1,820 events on Virus Counter® (Figure 5). Including buffer- and dye-only controls enabled accurate quantification of particles of interest in the purified EV sample, which showed substantially more events (8,433). Moreover, Sc-NTA and Fl-NTA showed the expected EV size distribution with a mean particle diameter of approximately 120 nm. All three techniques confirmed the particle concentration of the purified EV sample. Lastly, lysing with SDS confirmed the vesicular nature of the sample by reducing the particle concentration to approximately that of the dye-only sample as measured on Virus Counter® (Figure 5).

Next, the purified EV sample was investigated for purity, EV morphology, and protein profile. Purity, expressed as particles/mg total protein, increased 183-fold to a value of 8.01×10^{11} in the purified sample compared to the clarified conditioned medium (Figure 6). Cryo-EM showed intact vesicles with lipid bilayers, confirming purification of EVs. Next, Simple Western™ was used to evaluate the presence of canonical EV proteins. The EV tetraspanins CD9 and CD81, as well as the cytosolic protein syntenin–1, which is known to be recovered in EVs, were enriched in purified EVs compared to MSC lysate. Flotillin–1, another recovered cytoplasmic protein, was present in both MSC lysate and EVs.[4] Albumin was reduced in both samples. CD73 and CD105, canonical MSC markers, confirmed the MSC source of the EVs. Lastly, proteomics was performed using LC-MS to understand the broader purified EV protein profile. Proteomics identified additional EV-associated proteins such as PDCD6IP (ALIX) and various heat shock proteins.

Figure 6: Final product characterization using EM, Simple Western™, and proteomics

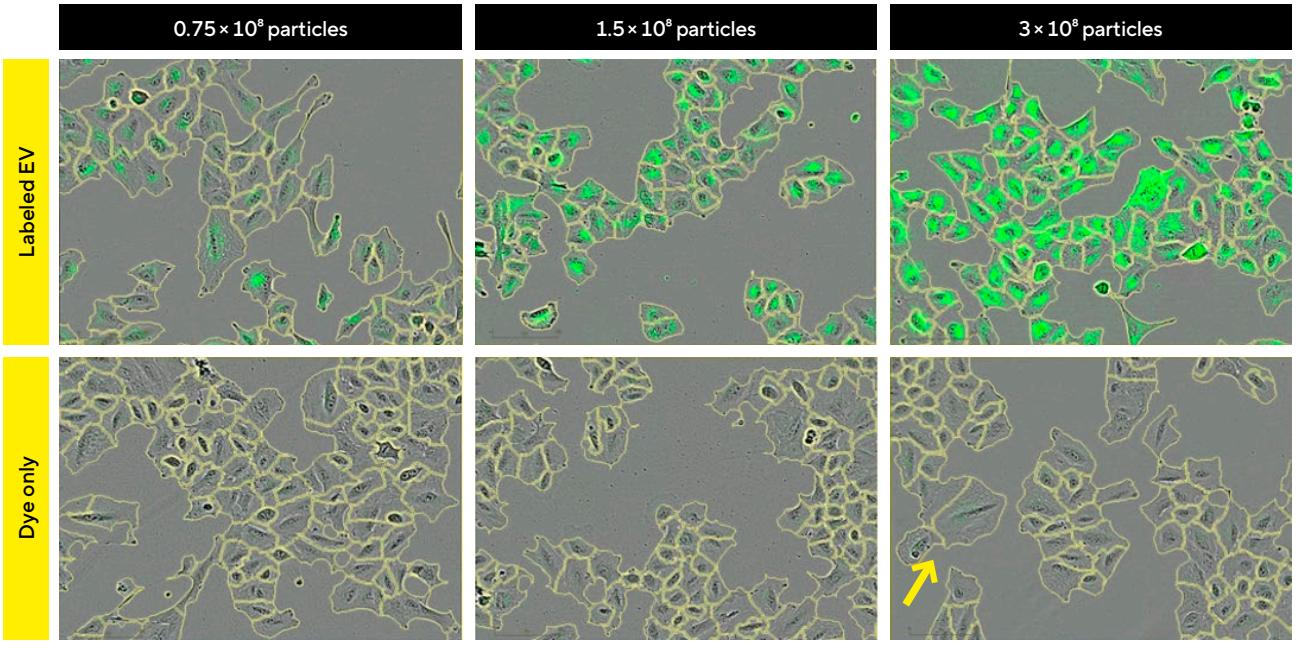


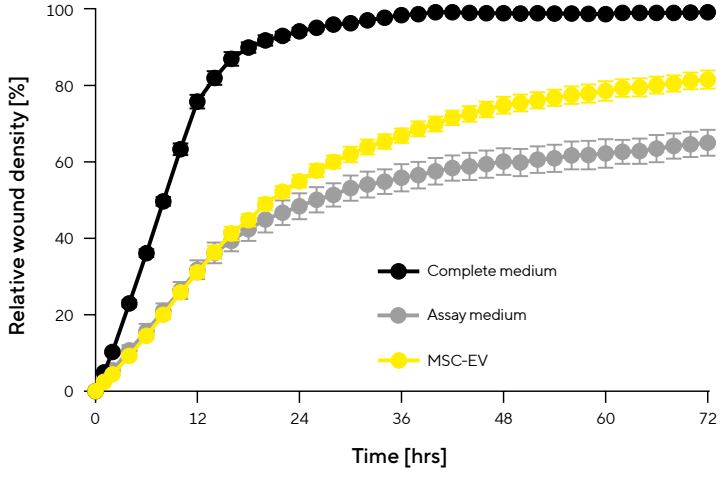
Final product function

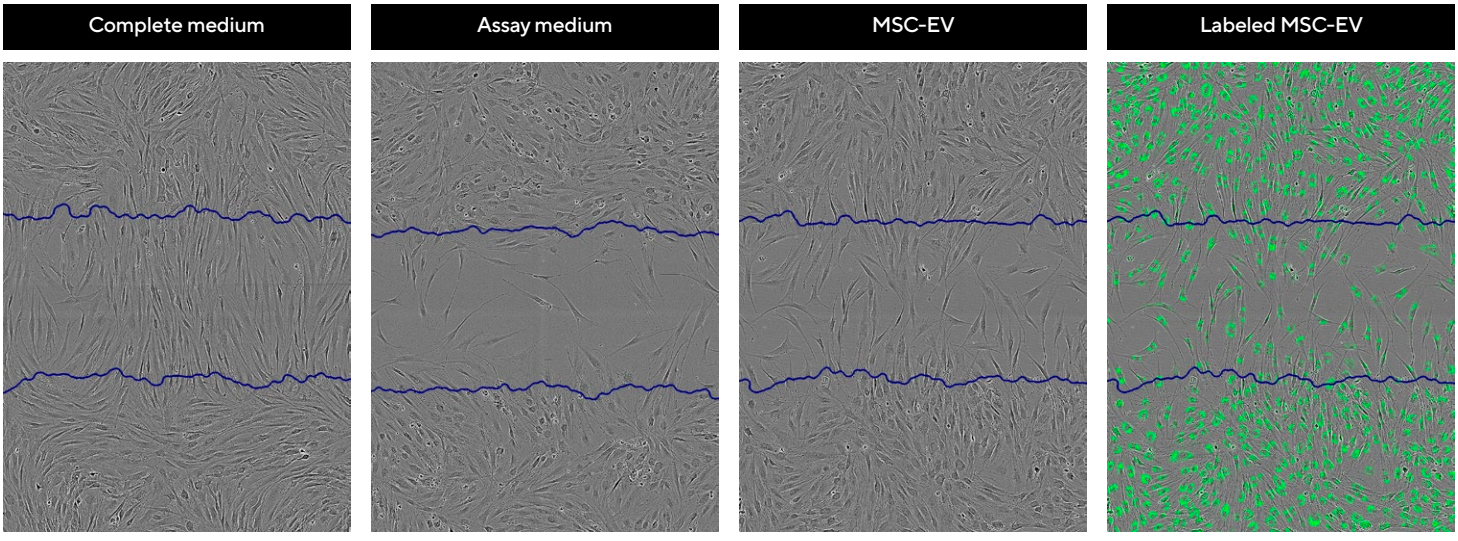
EV uptake by A549 cells over time was assessed using the Incucyte® cell imaging system (Figure 7). EVs were labeled using the Incucyte® Exofluor Green EV Labeling Kit. Labeled EVs were compared to a dye-only control. The labeled EVs qualitatively showed a dose-dependent increase in green fluorescence intensity from 0.75×10^8 to 3×10^8 particles per well without a significant increase in background fluorescence from the dye. Minimal background fluorescence at the highest particle dose is indicated by the yellow arrow. The yellow borders show cell segmentation from the Incucyte® Adherent Cell-by-Cell Analysis Software Module.

Since wound healing is a common therapeutic application of MSC-EVs, the purified EVs were tested in a fibroblast scratch assay.[5] Purified EVs added to normal human dermal fibroblasts significantly increased relative wound density compared to assay medium alone. Images at 18 h post-scratch qualitatively confirmed increased cell migration with EV treatment compared to assay medium alone, as well as substantial EV uptake using the Incucyte® Exofluor Green EV Labeling Kit (Figure 7). The blue line indicates the initial wound boundary in each image. Thus, EVs from the purification process demonstrated here retain bioactivity in fibroblast uptake and wound healing.

Figure 7: EV uptake using the Incucyte® Exofluor Green EV Labeling Kit and fibroblast scratch assay using the Incucyte® Live-Cell Analysis System







Conclusion

This application note outlines the development of a scalable end-to-end production process and a characterization platform for high-quality EVs from 3D-cultured hBM-MSCs using a comprehensive suite of Sartorius equipment and consumables. The process effectively integrates scalable EV production, clarification, TFF, and IEX chromatography to generate high-purity, high-yield EVs. Impurity reduction and product enrichment were demonstrated throughout the process, including through the process-monitoring capabilities of PATfix® analytical SEC. The orthogonal analytical techniques of NTA and nanoflow cytometry provided robust quantification of purified EV samples. Cryo-EM and Simple Western™ confirmed the quality of the purified EVs, while uptake and wound closure assays using the Incucyte® Live-Cell Analysis System confirmed that purified EVs retained bioactivity. Overall, this application note demonstrates an optimal approach for the manufacturing and analysis of EVs for therapeutic applications.

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Related Application Notes

Skerlos, L., Foltin, S., Campwala, H., Black, E., Dehghani, A., Bevan, N., & Schramm, C. (2024). *Harnessing exosomes for biomedical applications: Insights into extracellular vesicle labeling and cellular uptake using live-cell analysis.* [Application Note]. Sartorius. https://www.sartorius.com/en/products/live-cell-imaging-analysis/live-cell-analysis-resources/exosomes-ev-labeling-application-note

Related Publications

Dehghani, M., Chai, M., Talebloo, N., Morrissey, M., Larey, A., Keselman, P., Rana, A., Islam, M., Mayo, M., Speidel, J., Mukherjee, P., Zhao, Z., Haridas, N., Marks, P., Chon, J., Kishimori, E., Gaborski, T., Kim, Y., Joshi, D., … Olszowy, M. (2026). *Bioprocess design and optimization of extracellular vesicles derived from mesenchymal stromal cells. ACS Nano.* https://doi.org/10.1021/acsnano.5c19046

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